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Field Performance of Elite Transgenic Maize Plants Expressing an Insecticidal Protein Derived from *Bacillus thuringiensis*

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We introduced a synthetic gene encoding a truncated version of the CryIA(b) protein derived from *Bacillus thuringiensis* into immature embryos of an elite line of maize using microprojectile bombardment. This gene was expressed using either the CaMV 35S promoter or a combination of two tissue specific promoters derived from maize. High levels of CryIA(b) protein were obtained using both promoter configurations. Hybrid maize plants resulting from crosses of transgenic elite inbred plants with commercial inbred lines were evaluated for resistance to European corn borer under field conditions. Plants expressing high levels of the insecticidal protein exhibited excellent resistance to repeated heavy infestations of this pest.

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European corn borer (ECB), *Ostrinia nubilalis* (Hubner), is a major pest of maize in North America and Europe. Yield loss of 3 to 7% per borer per plant can result from ECB feeding at various stages of plant growth[1]. Feeding results in physiological disruption of plant processes, leading to lower plant yield. Yield reduction from ECB in Illinois corn is estimated to exceed $50 million annually[2]. Chemical pesticides are effective against ECB, but inconvenience of scouting fields and determining treatment thresholds, narrow application windows on large corn plants, as well as the behavior of the insect, generally result in poor control. ECB typically has two generations annually; however, three or four generations can occur in a large area of its North American distribution[3]. First generation egg masses are laid on the underside of corn leaves beginning in May. Newly hatched larvae migrate into the whorl and feed on leaf material for 7 to 10 days. Third instar larvae then tunnel into the stalk where they feed, pupate, and emerge as second generation moths over an extended period during midsummer. These moths deposit their egg masses on the underside of leaves, most often in the region of the ear node. After hatching, most neonate larvae move to the leaf axils to feed on pollen accumulated at these sites and on sheath and collar tissue. Once ECB larvae begin feeding inside the collar, they are protected from control by chemical pesticides. Larvae begin to tunnel into the stalk after three to six weeks, most often in the ear region, where their feeding can result in severe yield loss from stalk breakage and/or from ears dropping to the ground.

ECB is susceptible to various insecticidal crystal proteins, or δ-endotoxins, produced by a number of strains of *Bacillus thuringiensis*, a gram positive, spore forming soil microbe (for review, see ref. 4). These crystal proteins are typically produced as large protoxins that are solubilized in the insect digestive tract, where they are proteolytically cleaved to produce an active insecticidal protein. The activated protein binds specifically to receptors in the midgut of the insect and brings about lysis of the cells by formation of pores[5,6]. Insecticidal proteins from *Bacillus thuringiensis* have been expressed in plants to confer insect tolerance[7-11]. Such expression has proven difficult when the native genes from *Bacillus thuringiensis* were used, necessitating the use of a truncated version of the native lepidopteran active genes for measurable protein and insecticidal activity in the transgenic plant. Use of a native δ-endotoxin coding region, which has a high A-T content, appears to lead to abnormally low gene expression in plants. Plants in general have a higher G-C content than that found in the δ-endotoxins, with maize having an even more pronounced preference for high G-C content in coding regions[12]. Modifying the coding sequence to increase the G-C content of the native gene results in a dramatic increase in expression of the insecticidal protein[13]. Our attempts to express detectable levels of CryIA(b) protein in maize using a truncated version of the native coding sequence have not been successful; therefore a modified coding sequence was used.

Transgenic plants expressing insecticidal proteins derived from *Bacillus thuringiensis* have been field tested and shown to resist insect feeding[14,15]. Maize transformation has not, until recently, been a routine procedure. Hence production of transgenic maize containing an insecticidal gene from *Bacillus thuringiensis* has lagged behind production of more readily transformed dicot plants. We report here the field performance of elite hybrid maize plants containing a synthetic gene encoding a truncated version of the CryIA(b) protein derived from *Bacillus thuringiensis* var. kurstaki HD-1[16]. Expression of the synthetic *cryIA(b)* gene in transgenic maize was effected using either the cauliflower mosaic virus (CaMV) 35S promoter or a combination of the phosphoenolpyruvate carboxylase (PEPC) promoter and a pollen specific promoter, both from maize. Our transgenic plants produced high levels of insecticidal protein and exhibited excellent protection against extremely high, repeated infestations with ECB.

Results

Introduction of a synthetic *cryIA(b)* gene into maize. A synthetic version of the *cryIA(b)* gene (construction to be described elsewhere) was utilized in this study after attempts to express the native gene in maize failed to produce detectable levels of CryIA(b) protein. As reported by others[13], increasing the G-C content of a *Bacillus thuringiensis* insecticidal protein gene greatly enhances its expression in plants. Modification of the native *cryIA(b)* coding region, which has a G-C content of about 38%, to possess a G-C content of about 65% produced a gene which is expressed at a high level in maize. This version of the *cryIA(b)* gene has about 65% homology at the nucleotide level with the native gene and is designed to resemble a maize gene in terms of codon usage. To our knowledge, this is the most radical alteration of a δ-endotoxin sequence to date. The gene used in this study encodes the first 648 amino acids of the 1155 amino acid protoxin and the complete sequence is available upon

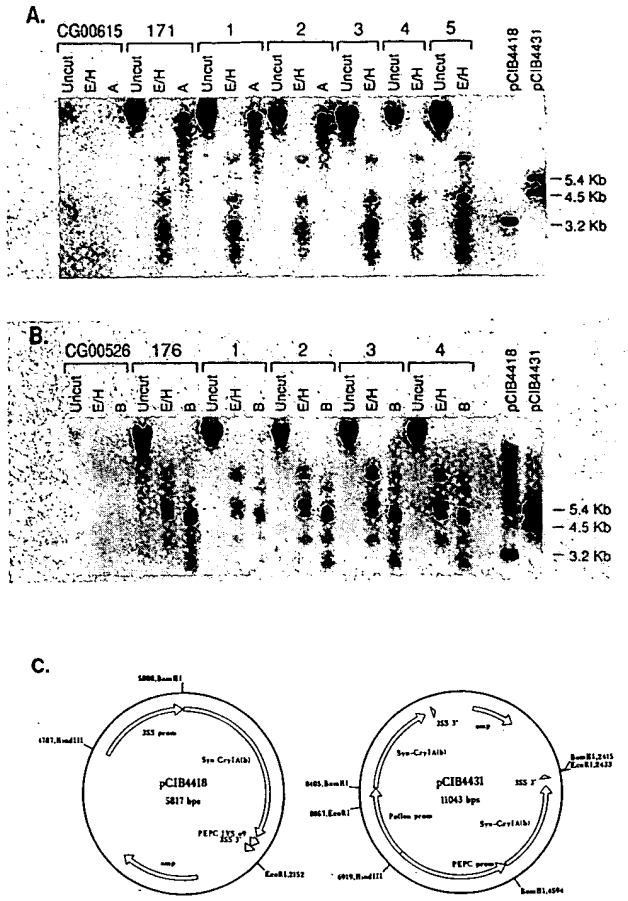

FIGURE 1. Southern blots of genomic DNA. The probe is specific for the synthetic *cryIA(b)* gene. (A) A parent plant and several progeny from event 171, the plants expressing the synthetic *cryIA(b)* gene using the CaMV 35S promoter. Lanes marked CG00615 contain DNA from a non-transgenic inbred with which the original transformants were crossed. Lanes marked 171: DNA from one of the original 171 regenerants. Lanes marked 1–5: DNA from five of the progeny from the 171 event. Lanes marked "uncut" are uncut DNA. Lanes marked E/H are cut with EcoRI and HindIII. Lanes marked "A" are cut with AflII. pCIB4418 is one of the plasmids used to transform the 171 event; it contains the 35S/syn-*cryIA(b)* gene. pCIB4431 is one of the plasmids used to transform the 176 event; it contains the PEPC/syn-*cryIA(b)* and pollen/syn-*cryIA(b)* genes. Both plasmids are cut with EcoRI and HindIII. (B) A parent plant and several progeny of the 176 event, the plants expressing the synthetic *cryIA(b)* gene using the PEPC and pollen promoters. Lanes marked CG00526 contain DNA from a non-transgenic inbred which was the recipient line for the transformation experiments. Lanes marked 176: DNA from one of the original 176 regenerants. Lanes marked 1–4: DNA from four of the progeny from the 176 event. Lanes marked "uncut" are uncut DNA. Lanes marked E/H are cut with EcoRI and HindIII. Lanes marked "B" are cut with BamHI. pCIB4418 and pCIB4431 are as described in (A) above. (C) Maps of the plasmids containing the synthetic *cryIA(b)* chimeric genes used to transform lines 171 (pCIB4418) and 176 (pCIB4431). The pollen/syn *cryIA(b)* and PEPC/syn *cryIA(b)* genes in pCIB4431 both contain the PEPC #9 intron in the 3′ untranslated region, as does the 35S/syn *cryIA(b)* gene.

request. The truncated protein encoded by this gene produces the same active insecticidal toxin as the full-length protoxin once it is proteolytically processed in the insect gut.

Chimeric *cryIA(b)* genes were introduced into a proprietary inbred line, CG00526, an elite cultivar of Lancaster parentage, using microprojectile bombardment of immature embryos, 14–15 days after pollination (see Experimental Protocol). Two independent events of transgenic maize expressing the synthetic *cryIA(b)* gene were chosen for further crossing and characterization. Initial transformants (multiple plants from each of the two events in this study) in the inbred line CG00526 were crossed to CG00526 as well as several other elite lines representing several heterotic groups. Event 171 contained the synthetic *cryIA(b)* gene under control of the CaMV 35S promoter and also contained a chimeric 35S/GUS gene for use as a scoreable marker. Event 176 contained a pair of tissue specific promoters controlling the expression of the synthetic *cryIA(b)* gene: the maize PEPC promoter[17], which is expressed in green tissues, and a maize pollen-specific promoter (to be described elsewhere). Both events contained a 35S/*bar* gene[18], used to confer resistance to phosphinothricin (PPT), as a selectable marker. The two events chosen for use in the field showed a 1:1 segregation ratio for phosphinothricin resistance, CryIA(b) expression, and ECB resistance when crossed with non-transformed plants. This observation, and other data (Southern analysis, Fig. 1), indicate a single site of insertion of the transgenes with a few copies of each gene. Parental and progeny plants from each event contain the same banding pattern in a Southern blot probed for the synthetic *cryIA(b)* gene. Uncut plasmids were used in the transformation experiments and therefore the banding pattern is more complex than that seen from cutting the plasmids themselves due to the random breakage of the plasmids prior to insertion into the plant genome.

Production of plants for field evaluation. Germination of immature embryos was employed to produce, in minimum time, the F1 hybrid plantlets for planting in the field. Immature embryos were germinated *in vitro* 14 to 15 days after pollination, allowed to develop, and transplanted to peat pots. When there was sufficient leaf material, samples were taken for analysis by β-glucuronidase (GUS) histochemical assay, PCR analysis for transgenes, growth in the presence of PPT, ELISA for CryIA(b) protein, and insect bioassay with ECB larvae. After analyses, plants were either discarded, shipped to Bloomington, Illinois, or retained in our greenhouse in North Carolina. Almost 1,000 plants were shipped to Illinois during June 1992, where the small peat pots containing the transgenic plants were transplanted into the field with 98% survival. Non-transgenic plants of inbred lines were planted in the same field over a six week period, starting in mid-May, to serve as controls and for pollinations.

Evaluation of transgenic maize plants in the field. When the plants reached approximately 40 cm in height, they were manually infested with neonate European corn borer larvae. About 300 larvae were applied to each plant per week for eight consecutive weeks for a total of 2,400 larvae per plant. The first four weeks of infestation correlated roughly with first generation timing and the second four weeks correlated with second generation infestation. ECB egg masses typically contain about 25 eggs. Thresholds of 0.5 egg mass per plant to as high as 4 egg masses per plant, depending on the relative tolerance of a particular maize hybrid, are generally used to determine if chemical control for second generation ECB is necessary. Assuming that every egg in an egg mass hatches, these 2,400 larvae would represent about 96 egg masses, half of which correspond to second generation infestation. Therefore our plants were challenged with 12 to 96 times the economic threshold of second generation ECB. The natural ECB pressure in surrounding fields was light and likely did not contribute significantly to the evaluation of these plants given the extremely high level of pressure from the repeated artificial infestations.

As indicated by the severe foliar and internal stalk damage

seen in the control plants, the ECB pressure we employed was more than adequate to evaluate the effectiveness of the synthetic *cryIA(b)* gene for ECB control. Table 1 and Figure 2 summarize the ECB damage ratings and their distribution for both first and second generation ECB infestations. All single cross transgenic families of both events were superior to the control inbred plants as assessed by either foliar feeding or internal stalk damage. With the exception of the CG00554 families, events 171 and 176 did not differ in performance for the above two traits. However, when averaged over all the families, event 176 derivatives had significantly better performance for foliar and internal stalk damage (data not shown). The average leaf damage rating for the best transgenic family, CG00554 X 176, was 1.6 compared to the non-transgenic inbred CG00554 which had a rating of 7.2.

Although first generation ECB control is important, the second generation can cause the greatest loss of yield, and is thus critical to the success of a commercial hybrid. The effectiveness of plants in controlling second generation larvae is ascertained by evaluating the extent of larval tunneling in the stalks. At the end of the field season, stalks from transgenic and control lines were split and examined for tunneling damage. The length of tunnels was significantly greater in control lines than in transgenic lines (Table 1 and Fig. 2). Transgenic plants expressing high levels of CryIA(b) protein had little or no tunneling damage. For example, the CG00554 X 176 family had an average of only 1.7 cm tunneling while the non-transgenic inbred had an average of 59 cm of tunneling damage. Typical differences in outer stalk damage and internal tunneling can be seen in Figure 3, as can the dramatic difference between transgenic and non-transgenic plants at the end of the growing season.

TABLE 1. Mean damage rating for first generation (ECB1) and second generation (ECB2), presented as foliar damage rating and tunnel length, respectively.

Maize Genotype	N	Mean ECB1 Foliar Damage Rating (1–9)[a]	Mean ECB2 Tunnel Length (cm)
CG00642 (Control)	10	6.3	40.7
CG00642 X 171	8	3.5	6.3
CG00642 X 1 76	13	3.0	2.7
CG00561 (Control)	10	7.3	60.8
CG00561 X 171	16	2.6	3.6
CG00561 X 176	7	2.1	2.3
CG00554 (Control)	10	7.2	59.3
CG00554 X 171	19	3.7	3.8
CG00554 X 176	13	1.6	1.7
CG00689 (Control)	5	7.1	28.3
CG00689 X 171	10	3.4	5.9
CG00689 X 176	8	2.8	2.4
CG00661 (Control)	5	6.7	113.8
CG00661 X 171	12	2.5	3.0
CG00661 X 176	14	2.9	3.9
CG00526 (Control)	19	6.2	41.3
CG00526 X 171	17	3.5	7.2
CG00526 X 176	15	3.5	5.2

N = number of plants characterized.
[a]Damage ratings were determined as follows:
1. No visible leaf injury.
2. Evidence of fine "window pane" damage only on the unfurled leaf where larvae plus corn cob grits fell into the whorl. No pin hole penetration of leaf.
3. Evidence of fine "window pane" damage on two unfurled leaves where larvae plus corn cob grits fell into the whorl. No pin hole penetration of leaf.
4. Evidence of pin hole or shot hole feeding damage that penetrated the leaf on two or more leaves that emerged from the whorl (any lesion < 0.25" in length).
5. Elongated lesions and/or mid rib feeding evident on more than 3 leaves that emerged from the whorl. Lesion < 1" in length.
6. Several leaves with elongated lesions (0.75" to 1.5" in length) and/or no more than 1 leaf with broken mid rib.
7. Long lesions (> 1") common on about one-half of leaves and/or 2 or 3 leaves with broken mid ribs.
8. Long lesions (> 1") common on about two thirds of leaves and/or more than 3 leaves with broken mid ribs.
9. Most leaves with long lesions. Several leaves with broken mid ribs. Possibly stunted plants due to ECB1 feeding.
Plants from event 171 contain the chimeric CaMV 35S/*cryIA(b)* gene while plants from event 176 contain the chimeric PEPC/*cryIA(b)* and pollen-specific/*cryIA(b)* genes.

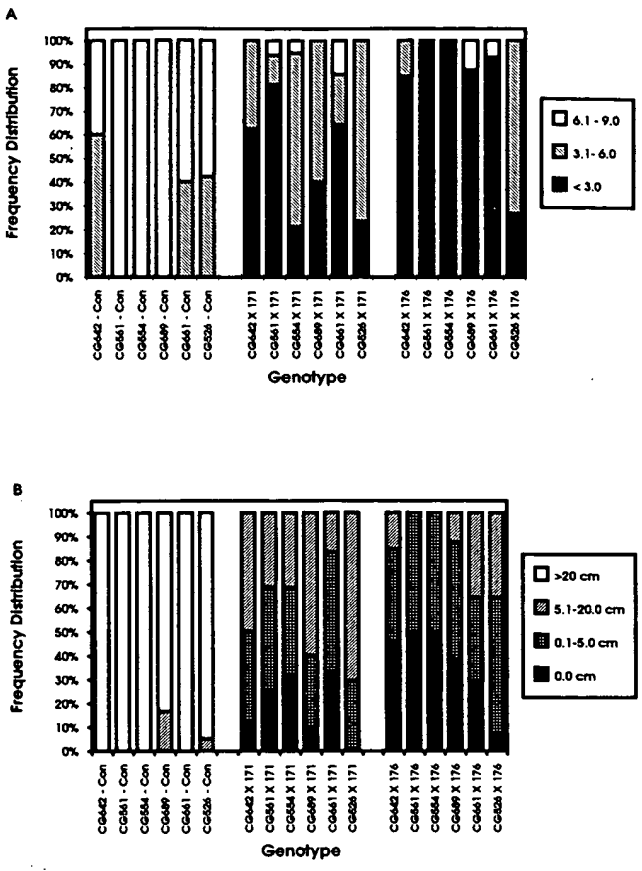

FIGURE 2. Frequency distribution of damage ratings for (A) first generation ECB (visual ratings 1–9) and (B) second generation ECB (tunnel length in cm). Event 171 = CaMV 35S/syn-*cryIA(b)* and event 176 = PEPC/syn-*cryIA(b)* and pollen/syn-*cryIA(b)*.

Laboratory assays were performed to determine the level of mortality brought about by ECB feeding on the transgenic plants. Leaf samples from the transgenic field plants were assayed for ECB activity by placing neonate larvae on leaf pieces in small petri dishes. Mortality was scored after 48 hours. All plants produced a high level of mortality within this time frame, with some at 100%. Typically, any larvae that were alive at 48 hours died by 72 or 96 hours. Results from some of these assays are shown in Table 2. We found a good correlation between the level of mortality at 48 hours and the level of CryIA(b) detected by ELISA (below). Plants with 100% mortality at 48 hours had the highest levels of CryIA(b) protein and also had the best overall performance ratings in the field.

Expression of chimeric *cryIA(b)* genes in maize. Transgenic plants with the best ECB damage ratings were analyzed for CryIA(b) protein levels using ELISA. Leaves from the field plants were sampled seven weeks post-transplant. Transgenic plants containing two synthetic *cryIA(b)* genes driven by the PEPC and pollen specific maize promoters produced over 1,000 ng CryIA(b)/mg soluble protein at week seven and were shown later in the season to exceed 4,000 ng

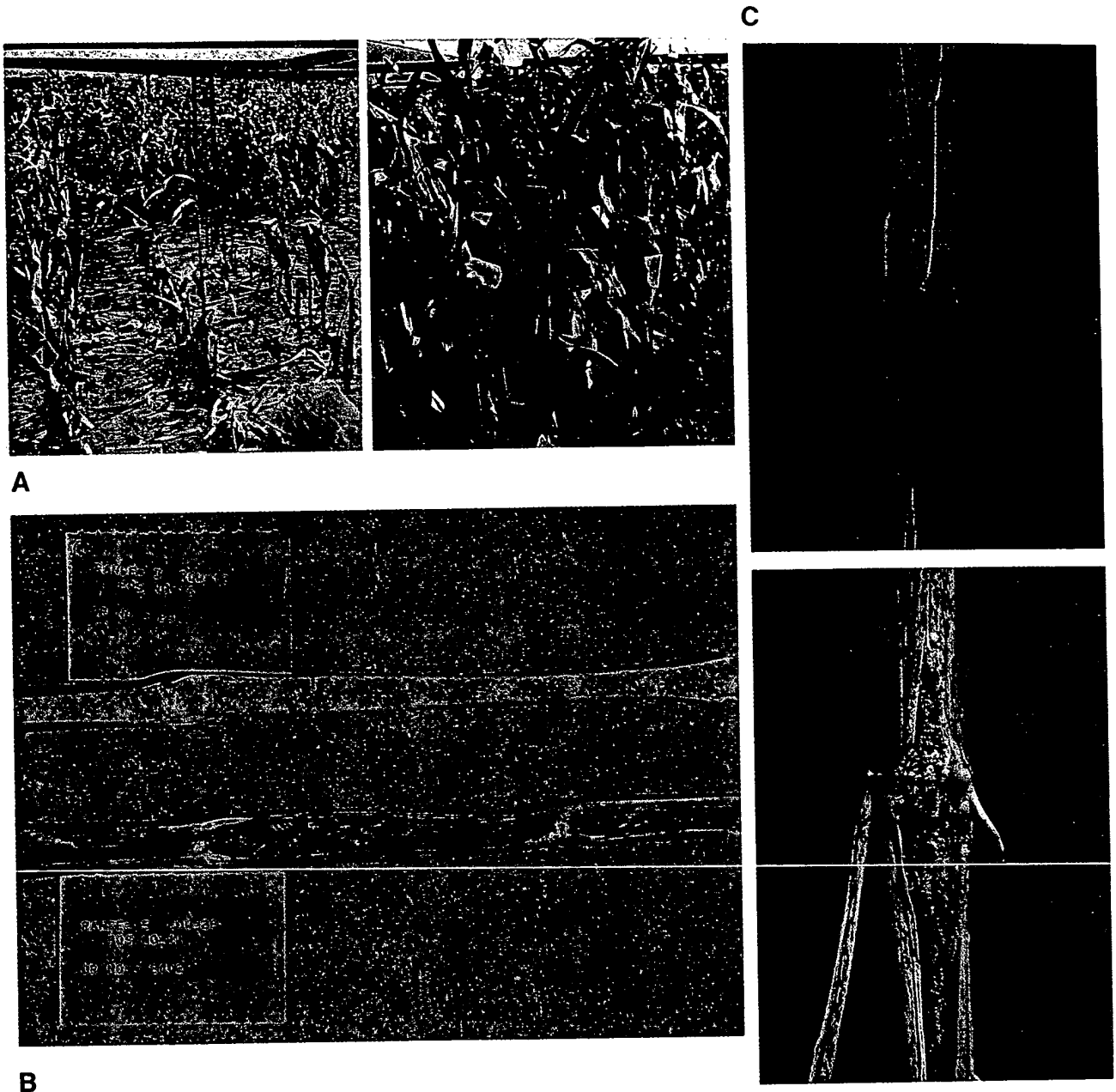

FIGURE 3. (A) View of non-transgenic (left) and transgenic (right) maize plants in the field at the end of the growing season. The ECB infestation pressure used to challenge these plants was high enough to cause complete loss of unprotected plants. (B) Split stalks from transgenic, upper, and non-transgenic, lower, plants showing the extent of tunneling damage caused by ECB in non-transgenic plants. (C) Outside view of stalks from transgenic, upper, and non-transgenic, lower, plants.

CryIA(b)/mg soluble protein. CryIA(b) protein levels in certain plants with the CaMV 35S promoter were as high at week seven as the levels in the PEPC and pollen promoter plants, but overall, these plants typically showed a much greater variation in CryIA(b) levels both within a particular cross with a given genotype and also among the genotypes. The levels of CryIA(b) in the PEPC and pollen promoter lines were all approximately equal in different genotypes. Table 3 presents typical ELISA values from five lines of each chimeric *cryIA(b)* construct.

Tissue specific expression patterns were evaluated by ELISA in several transgenic plants. Table 3 shows CryIA(b) protein concentrations in leaf, root, pith, pollen/anther, and kernels of selected plants. The CaMV 35S promoter is generally considered to be a constitutive promoter and as shown in Table 3, these plants had high levels of CryIA(b) in leaf, pith, and root. This correlates well with patterns of CryIA(b) expression observed in transgenic tobacco containing the same CaMV 35S promoter[11]. CryIA(b) was detected in the kernel but not in pollen/anther of the 35S plants. In the pollen and PEPC promoter plants, leaf expression of CryIA(b) was high while the pith and root expression levels were low. The green tissue surrounding the pith evidently contained sufficiently high levels of CryIA(b) to provide excellent insecticidal activity against ECB, as judged by the inability of ECB to penetrate the stalks significantly. While the level of CryIA(b) expressed in the pith of these plants was lower than that in the leaves, the level of expression was high enough to kill ECB, should any survive to penetrate the stalk. The high levels of CryIA(b) protein in pollen and low levels in kernel

produced by the combination of the PEPC and pollen promoters make this combination particularly attractive for producing a pattern of expression effective for controlling ECB while minimizing expression of CryIA(b) protein in seed and other tissues.

Discussion

Expression of a synthetic gene encoding a truncated version of the *cryIA(b)* insecticidal protein gene derived from *Bacillus thuringiensis* var. kurstaki HD-1 in elite single crosses of maize provides season long protection from repeated heavy infestations of European corn borer. Both first generation and second generation protection was obtained, even with an extremely heavy ECB infestation rate. Protection was obtained with plants hemizygous for the *cryIA(b)* gene; thus hybrid maize plants with only one transgenic parent can be protected. This is similar to the observation that heterozygous tobacco plants expressing a *cryIA(b)* gene were resistant to insects in a field situation[11,15]. Typical levels of CryIA(b) seen in plants containing the tissue specific chimeric genes were about 1,500 ng CryIA(b)/mg soluble protein, with levels as high as 4,000 ng/mg protein observed. The leaves in this test averaged about 8.5 mg total protein per gram fresh weight, so the plants were producing CryIA(b) protein at a "typical" level of 12,750 ng per gram fresh weight. The LC_{50} for ECB neonates is 20–30 ng/g diet. Adequate expression of CryIA(b) protein in pollen is important for control of second generation ECB larvae, for which pollen comprises a large portion of their diet during first and second instar. Transgenic plants containing the pollen and PEPC promoters expressing chimeric *cryIA(b)* genes produce the insecticidal protein in those parts of the plant consumed by both first and second generation ECB while minimizing expression in seed and other parts of the plants.

This is the first report of transformation of immature embryos from an elite maize inbred via microprojectile bombardment. Other reports of maize transformation have utilized suspension cultures[19,20] or callus cultures[21,22] of A188 X B73 crosses. Routinely, an average of one transformation event is recovered per 100 immature embryos bombarded, or one event for every 2.5 shots. The transgenic plants in this study were chosen from 23 events generated in 11 consecutive experiments. For the #171 event, 249 immature embryos were bombarded; 44 produced embryogenic callus on selection and the experiment yielded two transformation events. To obtain the #176 event, 251 embryos were bombarded, 37 of which produced embryogenic callus on selection. Although one embryo could in theory give rise to more than one transformation event, only one event is maintained from an embryo. Introduction of chimeric genes directly into elite inbreds could represent a significant savings in breeding time required to produce a commercial hybrid. As improved insecticidal genes become available, they can be introduced rapidly into commercial hybrids. The protection afforded by expression of the synthetic *cryIA(b)* gene in maize is significantly above that found thus far using traditional breeding methods. The ability to introduce improvements into elite inbreds, improvements which may not be within the genetic potential of the species, increases the alternatives available for management of pest problems and the rapidity with which they can become available.

The work presented here marks the first field evaluation of commercially relevant transgenic hybrid maize plants. One of the crosses made and evaluated employed the genotypes used in a commercial hybrid. Further, the plants tested represent two strategies for expression of foreign genes in plants for insect control. One strategy uses an essentially constitutive promoter, the CaMV 35S promoter, while the other uses conspecific tissue

TABLE 2. Percent mortality of ECB on leaf pieces from field grown transgenic maize.

Genotype	N[a]	Mean[b]	Range
35S Lines			
CG00642 X 171-4A	5	96	(85–100)
CG00554 X 171-4A	5	91	(75–100)
CG00526 X 171-4A	9	71	(70–100)
CG00661 X 171-4A	3	95	(90–100)
CG00554 X 171-4B	6	71	(55–90)
CG00689 X 171-13	7	75	(60–90)
CG00554 X 171-13	6	74	(55–100)
CG00561 X 171-14A	6	94	(85–100)
CG00689 X 171-14A	5	84	(70–90)
CG00554 X 171-14A	4	81	(70–95)
CG00716 X 171-14B	4	69	(50–80)
CG00615 X 171-15	2	90	(90)
CG00554 X 171-15	3	75	(55–95)
CG00526 X 171-15	1	65	(65)
CG00716 X 171-16AB	2	90	(80–100)
CG00689 X 171-16AB	2	68	(65–70)
CG00526 X 171-16AB	2	78	(70–85)
CG00661 X 171-16AB	5	99	(95–100)
CG00689 X 171-18	6	75	(65–90)
CG00554 X 171-18	3	72	(50–85)
CG00526 X 171-18	1	95	(95)
PEPC/Pollen Lines			
CG00689 X 176-11	5	96	(90–100)
CG00554 X 176-11	4	96	(85–100)
CG00526 X 176-11	2	100	(100)
CG00661 X 176-11	8	99	(95–100)
CG00642 X 176-10	2	100	(100)

[a]N = Number of plants assayed using 20 neonate larvae per plant
[b]Mean mortality was scored at 48 hours. Control plants produce an ECB mortality of 0–20%. Plants from event 171 contain the chimeric CaMV 35S/*cryIA(b)* gene while plants from event 176 contain the chimeric PEPC/*cryIA(b)* and pollen-specific/*cryIA(b)* genes.

TABLE 3. Quantification of CryIA(b) protein levels in various tissues of maize.

Genotype	Leaf	Root	Pith	Pollen/ Anther	Kernel
35S Lines					
CG00554 X 171-18	513 ± 244 (n=4)	596	288	NT	53
CG00554 X 171-4A	1732 ± 39 (n=2)	NT	NT	NT	NT
CG00554 X 171-13	767 ± 842 (n=8)	1209	4381	0	274
CG00554 X 171-1 4A	655 ± 554 (n=7)	3348	2440	0	149
CG00615 X 171-1 6BB	283 ± 227 (n=7)	74	71	0	NT
PEPC/Pollen Lines					
CG00661 X 176-10	1703 ± 378 (n=9)	52	60	260	15
CG00554 X 176-11	1288 ± 583 (n=12)	54	113	418	16
CG00642 X 176-10	1138 ± 188 (n=4)	NT	NT	NT	NT
CG00689 X 176-11	1077 ± 108 (n=3)	NT	NT	340	NT
176-11 X CG00526	1842 ± 345 (n=2)	47	53	NT	18

Values are ng CryIA(b)/mg soluble protein ± standard deviation.
Values for control plants analyzed by ELISA are 0 ng.
Plants from event 171 contain the chimeric CaMV 35S/*cryIA(b)* gene while plants from event 176 contain the chimeric PEPC/*cryIA(b)* and pollen-specific/*cryIA(b)* genes.
NT = not tested

specific promoters. Both types of plant produce high levels of CryIA(b) insecticidal protein. The plants containing chimeric insecticidal genes expressed using tissue specific promoters produce a consistently higher level of the CryIA(b) protein, but one must be mindful that these are only single transformation events leading to each of the different lines. Further events are under analysis to see how broadly these findings can be applied. Nonetheless, it is clear that expression of a high level of insecticidal proteins derived from *Bacillus thuringiensis* can protect maize plants from insect attack in a field environment.

Transgenic plants expressing insecticidal genes present a new tool for use in an integrated pest management strategy. Transgenic plants deliver the δ-endotoxins in a manner quite different from that of *Bacillus thuringiensis* microbial sprays. The larvae are exposed to the insecticidal protein in a more uniform manner on a constant basis and from the earliest possible time. The neonate larvae are the most susceptible stage and are exposed as soon as they begin to feed on the plant. They do not have an opportunity to avoid ingesting the insecticidal protein. As with any other insect control mechanism, development of resistance to the expressed insecticidal proteins might be a possibility. There are various strategies for preventing, or at least delaying, the onset of such resistance. As is true for any other insect control method, use of transgenic plants should represent only part of the total insect control practice. As agricultural biotechnology continues to mature, new strategies for insect control will become available. These will undoubtedly entail different mechanisms for insect control than are presented by the *Bacillus thuringiensis* δ-endotoxins. Use of a variety of such genes, either in a linear progression, on a revolving basis, or in various combinations, should combat the development of resistance to any single mechanism or gene product.

Experimental Protocol

Transformation vectors. Vectors used to transform maize are all derivatives of pUC18 or pUC19[23]. pCIB4418 contains a synthetic gene encoding the amino terminal 648 amino acids of CryIA(b) from *Bacillus thuringiensis* var. kurstaki HD-1[16], fused with the CaMV 35S promoter[24]. The sequence of this synthetic gene is available upon request from the corresponding author. pCIB4431 contains two chimeric *cryIA(b)* genes. The first is under control of the maize PEPC promoter[17] and the second is under control of a pollen specific promoter derived from maize (cloning and characterization to be described elsewhere). pCIB3064 contains a plant expressible *bar* gene[18] driven by the CaMV 35S promoter to provide resistance to phosphinothricin. pCIB3007 contains a GUS gene under control of the CaMV 35S promoter with a 144 NT leader derived from the CaMV 35S transcript (A. Montoya, unpublished data). A copy of the maize Adh1 intron #1 has been inserted into the 5′ leader of this gene.

Transformation and embryo rescue of progeny. Immature embryos (1.5 to 2.5 mm in length) of Ciba maize inbred CG00526, developed from a Lancaster-type population, were aseptically excised 14–15 days after pollination from surface-sterilized, greenhouse-grown ears and plated scutellum up on callus initiation medium, 2DG4 + 5 mg/l chloramben. 2DG4 medium is Duncan's "D" medium[25] modified to contain 20 mg/l glucose. Plasmid DNA was precipitated onto 1 μm gold microcarrier as described in the DuPont Biolistic manual. For event 176, 6.15 μg of pCIB4431 and 1.80 μg of pCIB3064 were used per 50 μl of microcarrier. For event 171, 2.72 μg of pCIB4418, 1.8 μg of pCIB3064, and 2.72 μg of pCIB3007 were used per 50 μl of microcarrier. Thirty-six embryos per plate were bombarded using the PDS-1000He Biolistic device. Tissue was placed on the shelf 8 cm below the stopping screen shelf and a 10 × 10 μm stainless steel screen was used with rupture discs of 1550 psi value. After bombardment, embryos were cultured in the dark at 25°C for one day, then transferred to callus initiation medium containing 3 mg/l PPT and incubated in the dark at 25°C. Resultant embryogenic tissue was transferred to callus maintenance medium, 2DG4 + 0.5 mg/l (2,4-dichlorophenoxy)acetic acid (2,4-D) containing 3 mg/l PPT and subcultured every 2 weeks. Twelve weeks later, tissue was cultured at 25°C on a modified Murashige and Skoog medium (MS)[26] containing 3% sucrose, 0.25 mg/l 2,4-D and 5 mg/l benzylaminopurine with 16 hours of light (50 μE/m-2/s-1) per day to initiate regeneration. Two weeks later the tissue was transferred to MS medium containing 3% sucrose. After 4 to 10 weeks, regenerated plants were cultured on MS medium modified to contain half the concentration of salts and 3% sucrose. Transformed plants were identified using the chlorophenol red (CR) assay to test for resistance to PPT[27], the histochemical GUS assay where appropriate[28] and PCR for sequences in the 35S promoter and the synthetic *cryIA(b)* gene. Positive plants were moved to the greenhouse for additional testing and crossing with various inbreds. Event 171 produced a total of 33 transgenic plants and event 176 produced a total of 38 transgenic plants. To minimize the time required to obtain T1 plants for field testing, we germinated immature embryos. Fourteen to sixteen days after pollination, the ear tip (25–50 kernels) was removed. The excised ear piece was surface-sterilized and individual embryos removed and plated on B5 medium[29] containing 2% sucrose. The method is described in detail by Weymann et al.[30].

Insect bioassays. When plants in the field reached about 40 cm of extended leaf height, infestation with laboratory-reared ECB larvae was begun on both the transgenic plants and non-transgenic controls. About 300 neonate larvae mixed with corn cob grits were introduced into the whorl of each plant using a Davis inoculator[31]. Infestations continued on a weekly basis for four weeks to simulate first generation corn borer (ECB1). Starting two weeks after the initial infestation, each plant was rated weekly for four weeks using a modification of the 1 to 9 scale described by Guthrie[32]; we reserved a rating of 1 for plants with no damage whatsoever. See legend to Table 1 for details. A mean ECB1 damage rating score was calculated for each transgenic plant and non-transgenic control plant. As each plant reached anthesis, 300 larvae/plant were applied weekly for four weeks to simulate second generation infestation (ECB2). One hundred neonate larvae in corn cob grits were introduced into the leaf axil at the primary ear and at the leaf axil one node above and below the primary ear node. About 50 days after the initial ECB2 simulated infestation, selected stalks from each of the two events in all lines as well as non-transgenic control plants were harvested. The extent of internal ECB tunneling damage in a 92 cm section of stalk 46 cm above and below the primary ear node was measured on a subset of transgenic and control plants. European corn borer assays using pieces of leaf from field plants were carried out in the laboratory. One to four 4 cm sections were cut from an extended leaf and placed on a moistened filter disc in a 50 X 9 mm petri dish. Five neonate European corn borer larvae were placed on each leaf piece and the petri dishes were incubated at 29.5°C. Leaf feeding damage and larval mortality data were scored after 48 hours.

CryIA(b) protein quantification. Detection and quantitative determination of the amount of CryIA(b) protein expressed in transgenic plants was monitored using enzyme-linked immunosorbant assays (ELISA)[33]. Immunoaffinity purified polyclonal rabbit and goat antibodies specific for the insecticidal crystal proteins from *Bacillus thuringiensis* subsp. kurstaki HD-1 were used to determine ng CryIA(b) per mg soluble protein from crude extracts of leaf samples. The sensitivity of the double sandwich ELISA is 1–5 ng CryIA(b) per mg soluble protein using 50 μg of total protein per ELISA microtiter dish well. Corn tissue extracts were prepared by grinding leaf tissue in gauze lined plastic bags using a hand held ball-bearing homogenizer (AGDIA, Elkart, IN.) in the presence of extraction buffer (50 mM Na_2CO_3 pH 9.5, 100 mM NaCl, 0.05% Triton, 0.05% Tween, 1 mM phenylmethylsulfonyl fluoride and 1 μM leupeptin). Protein determination was performed using the Bio-Rad (Richmond, CA) protein assay.

Statistical analysis. Analyses of variance were performed to compare the two events and to compare them with the control plants for the average foliar feeding damage ratings and for cm internal tunneling damage in the 92 cm stalk section using the general linear models (GLM) procedure in SAS[34] with the associated least significant differences (LSD) function. Similarly, the events and controls were compared to each other within all families.

Southern blot analysis. Genomic DNA was isolated from maize plants and processed for Southern blot analysis using standard procedures[35]. A gel purified fragment containing only the synthetic *cryIA(b)* gene was used to generate a random-primed ^{32}P probe. Southern blots were prepared and hybridized using standard procedures and washed at 65°C in 0.3 X SSC.

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